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Enzymic Synthesis of Deoxyribonucleic Acid

XVIII. The Repair of Partially Single-stranded DNA Templates by DNA Polymerase

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A partially single-stranded DNA, prepared by limited digestion of each strand with exonuclease III, can be restored to its native, fully double-stranded structure by *Escherichia coli* DNA polymerase. The rate of synthesis observed in the repair of such a partially degraded primer in the polymerase system is faster than that seen with a native DNA. The newly synthesized DNA is covalently attached to the primer. The fully repaired DNA resembles the original native DNA as judged by its appearance in electron micrographs, CsCl density-gradient analysis, denaturability and genetic activity. DNA synthesis which follows the repair phase produces a structure that is not covalently linked to the primer and resembles, in its nondenaturability, branched appearance, and lack of genetic activity, the product obtained with a native DNA primer (Schildkraut, Richardson & Kornberg, 1964).

1. Introduction

Native DNA as a primer for the DNA polymerase from *Escherichia coli* promotes an extensive synthesis of DNA. The product, indistinguishable from the primer by chemical and most physical analyses, has two unusual features. Synthetic DNA is nondenaturable, i.e. it readily reassumes a helical conformation after denaturing treatments (Schildkraut, Richardson & Kornberg, 1964), and it appears highly branched in electron micrographs (Inman, Schildkraut & Kornberg, unpublished work). Studies of these two characteristics of the enzymic product have suggested that they are associated with new strands that can be separated from the native template.

The complexity of the replication of a native template reflected in the structural anomalies in the product applies also to the response by *E. coli* polymerase to denatured (single-stranded) templates (Schildkraut *et al.*, 1964). Are these complications an inevitable consequence of polymerase action, or do they reflect the heterogeneity and complexities of the primer? An opportunity to evaluate this question was furnished by the use of exonuclease III to provide a unique population of primer molecules. Exonuclease III, the DNA phosphatase-exonuclease from *E. coli*, not only cleaves a 3′-phosphoryl monoester if present as a terminal group on a DNA chain (Richardson & Kornberg, 1964), but also splits the phosphodiester bonds successively from the 3′-hydroxyl terminus of a native DNA molecule (Richardson,

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Lehman & Kornberg, 1964*a*). Since this exonuclease action appears to be specific for a helical molecule, the product of its action may be regarded as a partially single-stranded structure as pictured in Fig. 1. The possibility that such an exonuclease III-degraded DNA might be used for replication by polymerase was indicated by earlier studies, which demonstrated that monomeric units were attached covalently to the

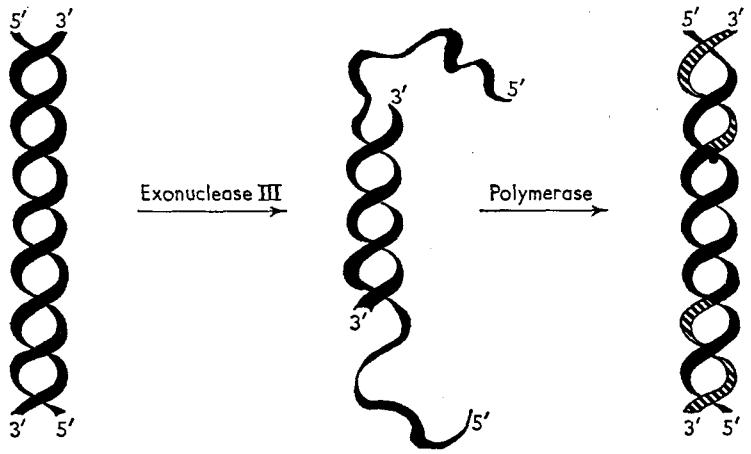

FIG. 1. *Postulated scheme for the polymerase repair of DNA partially degraded by exonuclease III.* The stepwise degradation from the 3′-hydroxyl end of each strand of native DNA by exonuclease III results in the formation of a partially single-stranded molecule. Synthesis by polymerase restores (striped sections) the original helical structure of the degraded DNA molecule.

primer (Adler, Lehman, Bessman, Simms & Kornberg, 1958) and that this reaction was dependent on base-pairing with a template (Schachman, Lehman, Bessman, Simms & Kornberg, 1958; Kornberg, 1961). In this sense then, the 3′-hydroxyl end of each strand which had been reduced in size by exonuclease III would serve as a primer, and the partially single-stranded portion of each strand would serve as a template to guide and dictate the synthesis (Fig. 1). This replication by polymerase to extend and complete a strand would, according to this model, represent a *repair* of the degradation by exonuclease III.

This paper presents evidence that *E. coli* DNA polymerase carries out the repair of a partially single-stranded DNA as described by the model in Fig. 1. The product is covalently attached to the primer strand, and upon completion of the repair a helical structure is restored which is fully denaturable, shows very few branches in electron micrographs and has all the properties that characterized the original native DNA.

2. Materials and Methods

(a) *Nucleic acids and nucleotides*

^{3}H-labeled DNA from T7 bacteriophage ([^{3}H]T7 DNA)† was isolated from phage grown in the presence of [^{3}H]deoxythymidine. *E. coli* B was grown at 37°C in 1 liter of the Casamino acid (Difco)–glycerol medium of Fraser & Jerrel (1953). Good aeration was achieved by using a 6-l. flask with rapid swirling on a gyratory shaker. At a cell titer of 2×10^9/ml., T7 phage were added at a multiplicity of 5 from a stock solution of

† Abbreviations used: [^{3}H]T7 DNA, ^{3}H-labeled DNA; [^{15}N^{2}H^{3}H]T7, ^{15}N^{2}H^{3}H-labeled DNA from T7 bacteriophage; dAT copolymer, the alternating copolymer of deoxyadenylate and deoxythymidylate; SSC, 0·15 M-NaCl containing 0·015 M-sodium citrate.

1×10^{12} phage/ml. 5 min later, 8 mc of [^{3}H]deoxythymidine were added and shaking was continued at 37°C for 1 hr to permit complete lysis. The lysate, containing $1{\cdot}7 \times 10^{11}$ phage/ml., was chilled to 4°C and all subsequent operations were carried out at this temperature. The phage were purified as described by Davison & Freifelder (1962) with the exception that three differential centrifugations of the phage solution preceded the banding in CsCl. The phage were recovered from the CsCl preparative gradient by collecting fractions dropwise from the bottom of the tube. The purified phage solution at an optical density of 17 at 260 mμ was dialysed for 12 hr against 0·10 M-potassium phosphate buffer, pH 7·45, containing 0·10 M-NaCl. The dialysed phage solution was shaken at 4°C with phenol saturated with a 0·10 M-potassium phosphate buffer, pH 7·45, containing 0·10 M-NaCl (Mandell & Hershey, 1960). Following a second extraction of the phage solution with phenol, the phenol layers were extracted twice with buffer and all of the aqueous layers were pooled. The DNA solution was dialysed extensively against the buffer and finally against 0·01 M-tris–HCl buffer, pH 7·8, containing 0·05 M-KCl. After phenol extraction of the DNA, only sterile solutions and glassware were used. From 1 liter of culture 19 μmoles of DNA-phosphorus were recovered. The extinction coefficient at 260 mμ ($O.D._{260}$) with respect to phosphorus was 6800 with a ratio of $O.D._{260}/O.D._{280}$ of 1·92. The buoyant density in CsCl of the native DNA was 1·710 g cm^{-3} and that of an alkali-denatured sample was 1·724 g cm^{-3}. The reduced specific viscosity (η_{sp}/c) was 79 dl./g in 0·02 M-KCl at a shear rate of approximately 80/sec. The specific activity of the DNA was $1{\cdot}6 \times 10^6$ cts/min/μmole of DNA-phosphorus.

[^{15}N^{2}H^{3}H]T7 DNA was isolated from bacteriophage grown according to the procedure described by Bodmer & Schildkraut (1964) for the preparation of ^{15}N^{2}H^{3}H-labeled T4 bacteriophage. Spontaneous lysis of the cells eliminated the necessity of chloroform treatment of the culture. The T7 phage were purified and the DNA was isolated in a manner identical to that described for the preparation of [^{3}H]T7 DNA. From 100 ml. of lysate ($2{\cdot}3 \times 10^{10}$ phage/ml.), 0·4 μmole of DNA-phosphorus was obtained. The extinction coefficient at 260 mμ with respect to phosphorus was 6850 with a ratio $O.D._{260}/O.D._{280}$ of 1·93. The buoyant density in CsCl of the native DNA was 1·758 g cm^{-3} and that of an alkali-denatured sample was 1·773 g cm^{-3}. The specific activity of the DNA was $1{\cdot}8 \times 10^7$ cts/min/μmole of DNA-phosphorus.

[^{3}H]*Bacillus subtilis* DNA (SB-19) (1×10^6 cts/min/μmole of DNA-phosphorus) isolated by the method of Marmur (1961), and [^{15}N^{2}H^{3}H]*B. subtilis* DNA ($2{\cdot}2 \times 10^6$ cts/min/μmole of DNA-phosphorus) prepared by the method of Bodmer & Schildkraut (1964), were gifts from Dr. Walter Bodmer. Calf thymus DNA was prepared by the procedure of Kay, Simmons & Dounce (1952).

Unlabeled deoxyribonucleoside and ribonucleoside triphosphates were purchased from the California Corporation for Biochemical Research. [2-^{14}C]-deoxythymidine, purchased from the New England Nuclear Corporation, was enzymically phosphorylated to dTTP as previously described (Okazaki & Kornberg, 1964).

Concentrations of DNA and RNA are expressed as equivalents of nucleotide phosphorus. Unless otherwise stated, all DNA samples used in this paper contained ^{3}H-label.

(b) *Enzymes*

The most purified fraction of DNA polymerase from *E. coli* (hydroxylapatite, fraction IX) was used in these studies. This fraction had a specific activity of 18,800 units/mg of protein and was prepared and assayed as described elsewhere (Richardson, Schildkraut, Aposhian & Kornberg, 1964*b*). Exonuclease I, the phosphodiesterase from *E. coli*, was the DEAE–cellulose fraction prepared and assayed as described elsewhere (Lehman, 1960). Exonuclease III, the DNA phosphatase-exonuclease from *E. coli*, was the phosphocellulose fraction prepared as described previously (Richardson & Kornberg, 1964). This preparation was assayed for its exonuclease activity as described previously (Richardson *et al.*, 1964*a*) and had a specific activity of 16,000 units/mg of protein.

(c) *Partial degradation of native DNA by exonuclease III*

Partially degraded DNA was prepared by incubating either native [^{3}H]T7 DNA or [^{3}H]*B. subtilis* DNA with exonuclease III at 37°C. The reaction mixture contained (per ml.)

270 mμmoles of DNA, 66 μmoles of tris–HCl buffer, pH 8·0, 1 μmole of $MgCl_2$, 10 μmoles of 2-mercaptoethanol, and 40 units of exonuclease III. The extent of degradation was determined by removing samples from the reaction mixture and assaying for the production of acid-soluble radioactive material. To 0·01 to 0·10 ml. of the reaction mixture, cold water was added to a volume of 0·30 ml., followed by the addition of 0·20 ml. of carrier calf thymus DNA (2·5 mg/ml.) and 0·50 ml. of cold 10% trichloroacetic acid. After 5 min at 0°C, the precipitate was removed by centrifuging for 10 min at 10,000 *g* at 4°C, and 0·50 ml. of the supernatant solution was pipetted into a glass bottle. 20 ml. of a scintillator solution consisting of 7 gm of 2,5-diphenyloxazole (PPO), 50 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene (dimethyl POPOP), and 200 g of naphthalene per liter of dioxane were added to the bottle and the sample was counted in a Packard Tri-carb liquid-scintillation counter. When the desired amount of hydrolysis was reached, the enzyme was inactivated by heating the reaction mixture at 65°C for 5 min and then cooling to 0°C. (Both the phosphatase and exonuclease activities of the DNA phosphatase-exonuclease are inactivated (more than 95%) by heating at 60°C for 4 min in the standard reaction mixture. There is no restoration of either activity during a 5-hr incubation at 0°C or 37°C.)

(d) *Synthesis of DNA*

The reaction mixture contained (per ml.) 66 μmoles of potassium phosphate buffer, pH 7·0, 6·6 μmoles of $MgCl_2$, 1 μmole of 2-mercaptoethanol, 50 mμmoles each of dATP, dGTP, dCTP and [^{14}C]dTTP ($1·2 \times 10^7$ cts/min/μmole), 30 mμmoles of DNA†, and 6 units of polymerase. The reaction mixture was incubated at 37°C unless otherwise stated. The extent of synthesis† was measured by removing samples from the reaction mixture and assaying for the production of acid-insoluble radioactive material as described elsewhere (Schildkraut *et al.*, 1964).

A mixed polymer of ribo-and deoxyribonucleotides was synthesized as described by Berg, Fancher & Chamberlin (1963). The reaction mixture contained (per ml.) 66 μmoles of tris–HCl buffer, pH 7·4, 1 μmole of $MnCl_2$, 1 μmole of 2-mercaptoethanol, 75 mμmoles each of rCTP, dATP, dGTP and [^{14}C]-dTTP ($1·2 \times 10^7$ cts/min/μmole), 180 mμmoles of [$^{15}N^2H^3H$]*B. subtilis* DNA 30% degraded by exonuclease III, and 200 units of polymerase. The reaction mixture was incubated at 37°C and the extent of synthesis was determined as described above for the standard polymerase reaction.

(e) *Measurement of denaturability of DNA by its susceptibility to exonuclease I*

A rapid and quantitative assay for the denaturability of a DNA is provided by measuring its susceptibility to exonuclease I (Schildkraut *et al.*, 1964), an enzyme specific for single-stranded DNA (Lehman, 1960). The radioactively labeled DNA, either untreated or denatured with alkali, was incubated with 20 μmoles of glycine buffer, pH 9·2, 2 μmoles of $MgCl_2$, 0·5 μmole of 2-mercaptoethanol, and exonuclease I (2 units per mμmole of DNA) in a volume of 0·30 ml. After incubation at 37°C for 30 min, the reaction mixture was assayed for remaining acid-insoluble radioactive material as described elsewhere (Schildkraut *et al.*, 1964).

(f) *Electron microscopy*

The method used to visualize DNA molecules in the electron microscope was a modification of the technique recently described by Kleinschmidt, Lang, Jacherts & Zahn (1962) and will be described at another time (Inman, unpublished results). A Hitachi HU-10

† Unless otherwise stated, the amount of DNA added to the polymerase reaction mixtures refers to that amount initially present before degradation by exonuclease III. When the extent of synthesis is determined, it is expressed relative to the DNA present prior to exonuclease treatment. For example: 0·3 μmole of 1 μmole of DNA is converted to acid-soluble mononucleotides (30% degraded) by exonuclease III; the partially single-stranded product is subsequently used to prime a synthesis by polymerase to the extent that 0·3 μmole of nucleotide is incorporated into an acid-insoluble polymer. This represents a 30% synthesis based on the original 1 μmole of DNA present before exonuclease treatment.

electron microscope was used at an instrument magnification of 6400 and was operated at 50 kv. Micrographs were projected to a final magnification of 140,000 and the molecules were then traced onto paper and their length determined with a map measurer. Usually the micrographs were of low contrast and, accordingly, high-contrast contact negatives were made for projection.

Certain rules were followed in order to decrease any tendency to selection during photography of the macromolecules and subsequent determination of their lengths. Micrographs were taken of a consecutive set of random fields in which any DNA molecules could be observed. A particular field was ignored if it happened to contain objects likely to interfere with length determination. All molecules in all micrographs were measured unless: (a) a micrograph was not in focus; (b) the particular configuration of a molecule was so convoluted as to make a length determination ambiguous; (c) for any other reason a length determination would be ambiguous. An approximate count shows that only 5% of the total molecules were rejected because of rules (b) and (c).

(g) *Other materials and methods*

Methods for density-gradient centrifugation were as previously reported (Schildkraut *et al.*, 1964). CsCl, optical grade, was purchased from the Harshaw Chemical Company, Cleveland, Ohio. Phenol was Mallinckrodt Analytical Reagent grade liquified phenol, containing no preservatives, and was used without further purification. Inorganic phosphorus was determined by the method of Chen, Toribara & Warner (1956). Deoxypentose was determined by the method of Dische (1955).

Transforming factor assays for conversion of *B. subtilis* strain SB 202 to try_2^+ (indole) were carried out as described by Nester, Schafer & Lederberg (1963); we are grateful to Dr. Walter Bodmer, Department of Genetics, Stanford University, for carrying out these assays. A DNA concentration of 0·5 to 1·5 mμmoles/ml. and 2 to 5×10^8 competent cells/ml. were used in these experiments.

3. Results

(a) *Kinetics of repair of partially degraded DNA*

(i) *Priming by native and partially degraded DNA at 37°C*

Native T7 DNA was treated with exonuclease III in order to obtain primers degraded 10, 17 and 29%, that is, from which 10, 17 and 29% of the nucleotide residues had been removed. As shown in Fig. 2, these partially single-stranded molecules primed a more rapid initial rate of DNA synthesis than did the untreated control. Of particular interest was the point at which the rapid initial rate changed to one that was only one-fifth as great. This point in the biphasic curve corresponded to an extent of synthesis that correlated closely with the extent to which each of these DNA primers had been degraded by exonuclease. In the case of the 10% degraded primer, the change in rate occurred between 8 and 10% synthesis; with the 17% degraded primer between 10 and 20% synthesis; and with the 29% degraded primer between 25 and 30% synthesis.

B. subtilis DNA, partially degraded with exonuclease III, also showed this biphasic rate of DNA synthesis when used as a primer. With a 30% degraded *B. subtilis* DNA, there was an initial rapid rate approximately sixfold faster than the final linear rate of synthesis: the change in rate occurred between 15 and 30% synthesis (Fig. 11(b)).

(ii) *Priming by DNA degraded 1% by exonuclease III*

When the T7 DNA degraded only 1% by exonuclease III was used as primer in the same polymerase system, the biphasic curve obtained with the more extensively degraded primers was not seen. However, this DNA preparation promoted synthesis

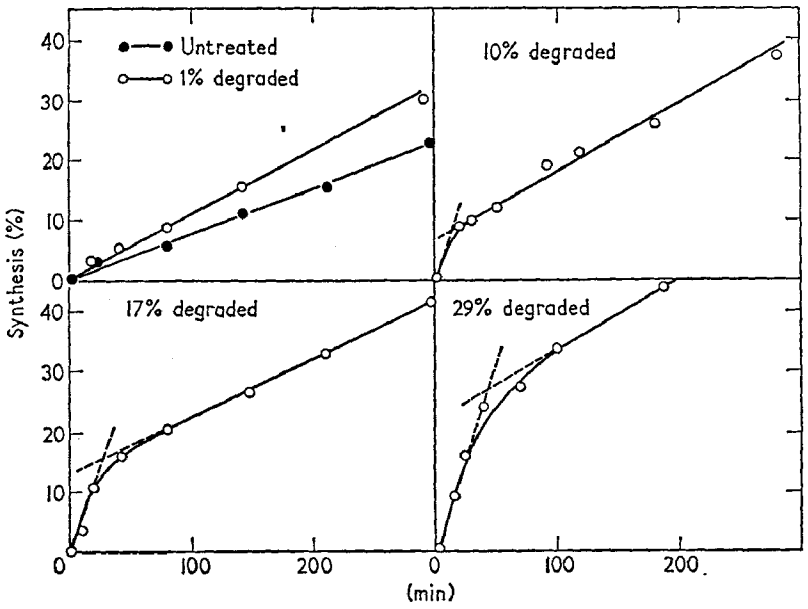

FIG. 2. *Kinetics of repair at 37°C of DNA partially degraded by exonuclease III.* T7 DNA was incubated with exonuclease III as described in Materials and Methods. Samples (0·30 ml.) were removed over a 3-hr period and the enzyme was inactivated by heating; the extent of degradation was determined by measuring the acid-soluble radioactive material produced (see Materials and Methods). The five samples included one taken before addition of enzyme (0%) and after degradation of 1, 10, 17 and 29%. DNA (30 mμmoles, see footnote, p. 49) from each of the five samples was incubated with polymerase and the extent of synthesis was determined by removing fractions and measuring the acid-insoluble radioactive material produced (see Materials and Methods). The percentage synthesis in each case was calculated relative to the amount of DNA present before degradation by exonuclease III (see footnote, p. 49).

at a rate approximately 1·5-fold faster than the untreated control (Fig. 2). This increased rate of synthesis is most likely due to the removal of inhibitory 3′-phosphoryl end-groups by the phosphatase action of the exonuclease III (Richardson & Kornberg, 1964; Richardson, Schildkraut & Kornberg, 1964*c*). Supporting this interpretation is the finding that the slower rate in the biphasic synthesis obtained with the more extensively degraded primers shown in Fig. 2 is nearly identical to the rate with the untreated DNA.

(iii) *Priming by native and partially degraded DNA at 20°C*

The priming by certain oligonucleotides for dAT synthesis is improved by lowering the temperature of the reaction, whereas the priming by dAT polymer is strongly decreased at the lower temperature (Kornberg, Bertsch, Jackson & Khorana, 1964). It appeared from these results that although the separation of the strands of a double-helical primer would be strongly affected by the temperature, the replication of a single-stranded template by polymerase might not be so affected. Figure 3 shows the effect on the rate and extent of synthesis with the native and partially degraded primers when the polymerase reaction was carried out at 20°C rather than at the routine temperature of 37°C. When native T7 DNA was added to a polymerase reaction mixture like that used in Fig. 2, a diminished rate of synthesis was observed which continued to the extent of only 3%. In contrast to the behavior of the native DNA,

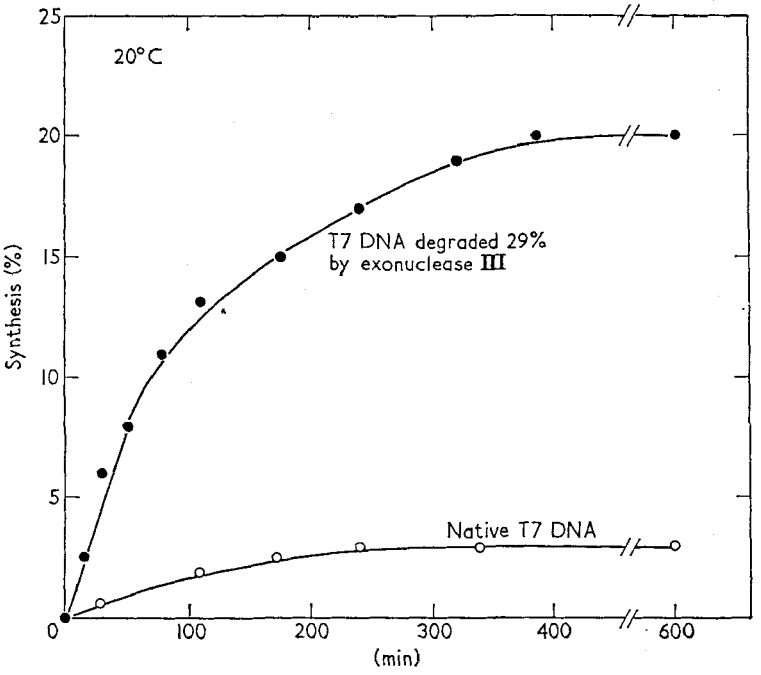

FIG. 3. *Priming by native and partially degraded T7 DNA at 20°C.* Untreated native T7 DNA and T7 DNA degraded 29% by exonuclease III (samples described in Fig. 2) were incubated with polymerase under the identical conditions described in Fig. 2, except that the reaction mixture was maintained at 20°C instead of 37°C. The amount of synthesis was determined by removing fractions and measuring the formation of acid-insoluble radioactive material as described in Materials and Methods.

the T7 DNA which had been previously degraded 29% by exonuclease III still permitted synthesis at an initial rate almost one-half that seen at 37°C. Furthermore, the reaction continued to the extent of 20% synthesis, beyond which no further synthesis was detectable.

These kinetic results suggest that polymerase is able to replicate the single-stranded regions of a DNA primer, created by exonuclease action, more rapidly than the intact native DNA molecule. Once synthesis has restored or repaired the degraded strand, polymerase is still able to utilize the double-stranded molecule as primer, but at a diminished rate. By lowering the temperature this difference in priming ability by partially single-stranded DNA and double-stranded DNA becomes more striking; the degraded DNA still functions as a primer up to the point where the single-stranded regions have been eliminated by the repair reaction, while the native DNA or repaired DNA is unable to promote synthesis at this temperature.

(b) *Structure of primer and products as revealed by electron microscopy*

The native T7 DNA (Plate I) exhibited a reasonably sharp distribution of molecular lengths (Fig. 4(a)) with the major peak at 10·9 μ. The number-average length for the total distribution was 9·1 μ (Table 1). The shorter molecules amount to 19% of the total mass of DNA; and at present it is not known whether these were present in the original solution or were produced by breakage of longer molecules during preparation for electron microscopy.

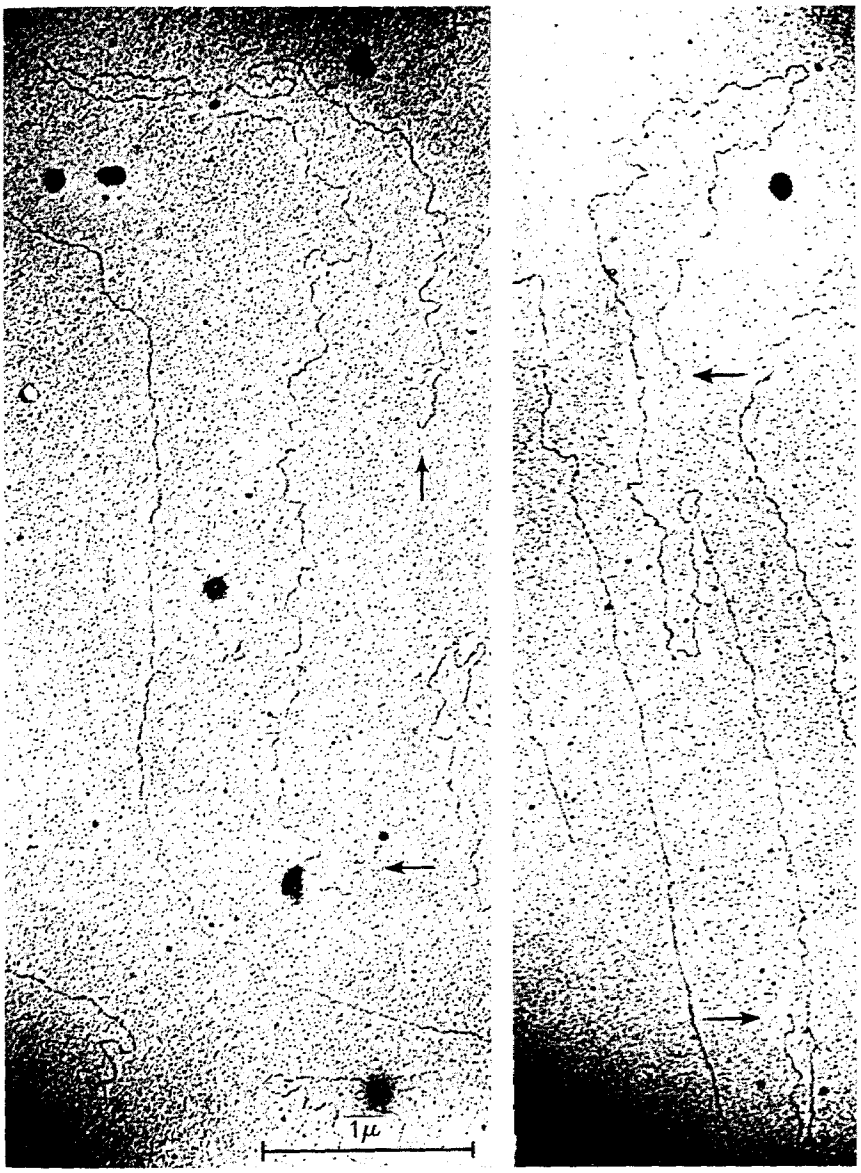

PLATE I. Electron micrographs of native T7 DNA. The length of unbroken T7 DNA molecules was 10·9 μ and the number-average length of the total population (L_N) was 9·1 μ.

[*facing p. 52*

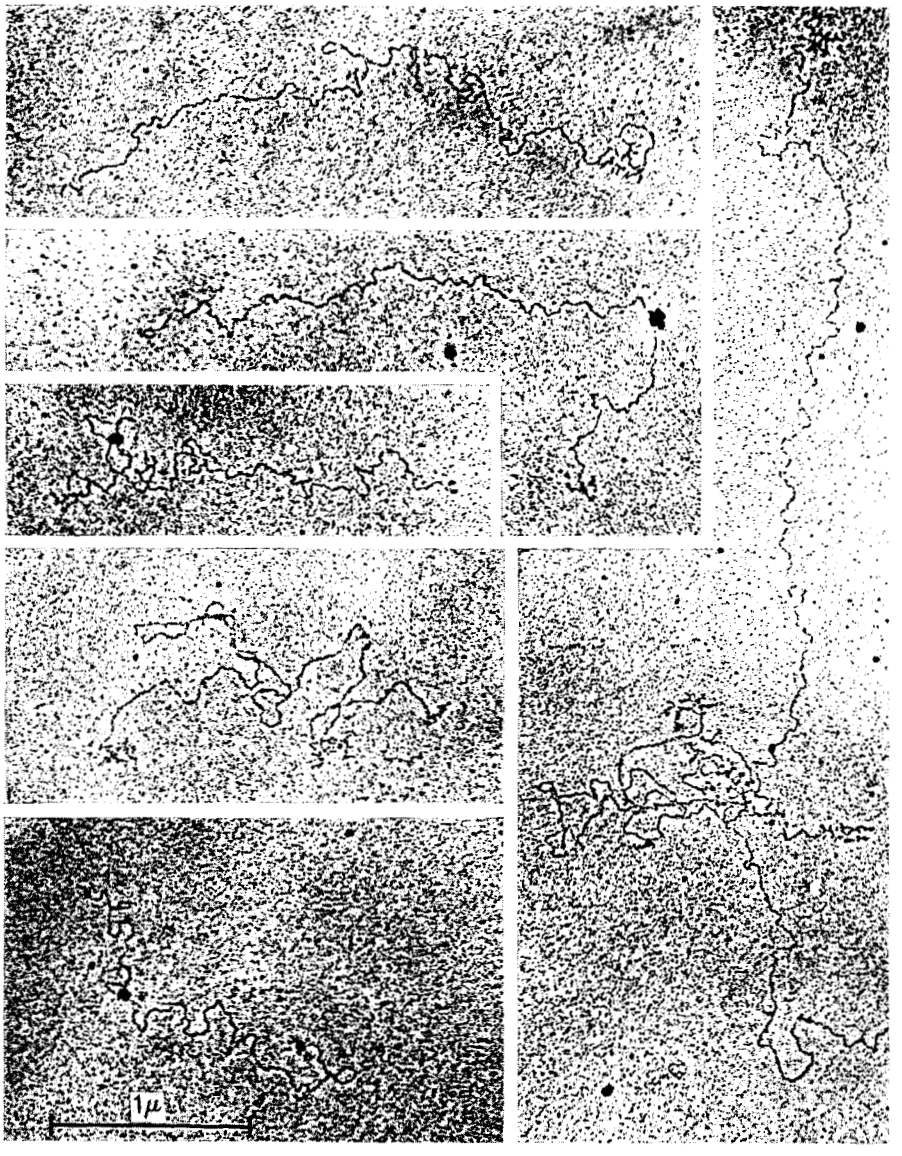

PLATE II. Electron micrographs of T7 DNA 17% degraded by exonuclease III showing: (a) decreased helical length ($L_N = 5{\cdot}9\,\mu$); (b) appearance of puddles at many of the ends; (c) tendency to aggregate in a small number of the molecules (far right micrograph).

(i) *Partially degraded T7 DNA*

T7 DNA samples degraded by exonuclease III to the extent of 17 and 29% showed a very much reduced length of helical structure (Fig. 4(b) and (c)) and in rough proportion to the extent of degradation (Table 1).

TABLE 1

Electron microscopic analysis of T7 DNA molecules

DNA	Number of molecules measured	Length (number-average) μ	Ends with puddles %	Branched molecules %
Native	31	9·1	< 2	0
Degraded 17% by exonuclease	33	5·9	60–70	0
Degraded 29% by exonuclease	98	2·4	30–40	0
24% synthesis with 29% degraded DNA	42	7·2	10–20	7
70% synthesis with 29% degraded DNA	34	8·0	0	56

The samples of T7 DNA analysed here by electron microscopy (see Materials and Methods) were the same as those cited in Fig. 4. See text for a description of puddles and branches.

Micrographs of degraded DNA often show a "puddle" at one or both ends of the molecule (Plate II). Puddles were observed at about one-third of the ends of a 29% degraded sample, whereas they were rarely if ever seen in the original DNA (Table 1). Identification of puddles is dependent upon the electron micrograph preparation. As shown in Table 1, puddles were visualized in 60 to 70% of the 17% degraded sample. An examination of these puddles at higher specimen resolutions, prepared by the method suggested by Bendet, Schacter & Lauffer (1962) and shadowed, without rotation, with a minimum amount of platinum, indicated that, although the puddles do resemble non-helical DNA, they appear also to contain many small globular impurities which contribute to the appearance of the puddles seen in the micrographs at lower resolution (Plate II). This phenomenon may be responsible, in part, for the variability in the number of puddles visualized in different electron micrographs of partially degraded DNA. The reduced lengths of helical DNA and the terminal regions of non-helical DNA support the hypothesis in Fig. 1 that the release of acid-soluble material during exonuclease III degradation has left single-stranded regions at each end of the molecule. 16% of the degraded molecules contained an interior puddle. One possible cause of such subterminal single-stranded regions may be degradation initiated at a single-stranded break in the primer. Another possibility is a tendency of single-stranded regions to aggregate with other molecules (Plate II). Aggregation was not observed in micrographs of the native DNA.

(ii) *Repair of partially degraded T7 DNA*

T7 DNA 29% degraded by exonuclease III was used to prime a polymerase reaction (see Fig. 2) and a sample was removed at 24% synthesis; at this point 83%

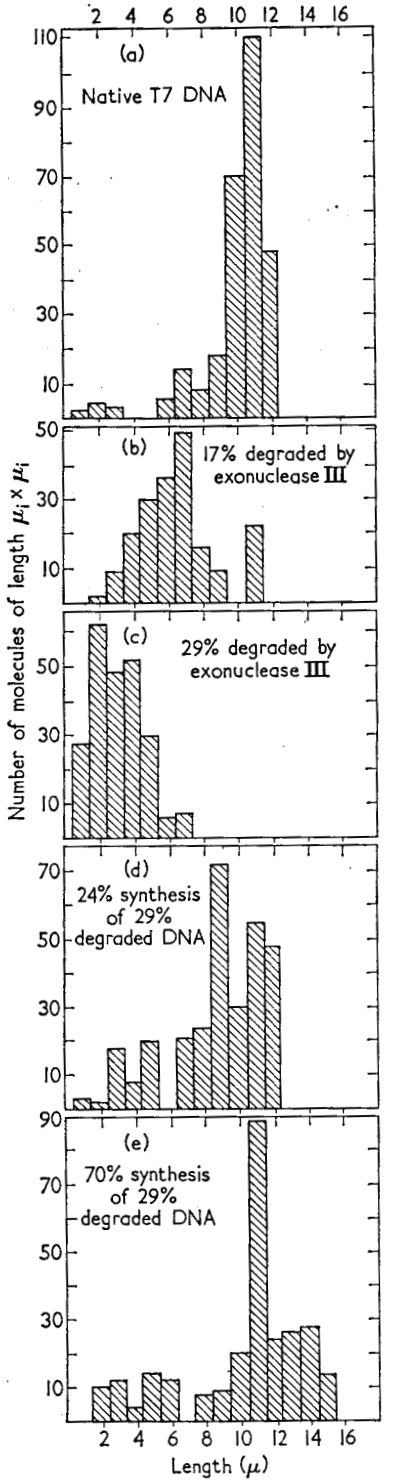

FIG. 4. *Distribution of lengths obtained by electron microscopy of T7 DNA samples before and after treatment with exonuclease III and polymerase.* Native T7 DNA (a), was 17% degraded (b), and 29% degraded (c) by exonuclease III. The 29%-degraded T7 DNA was incubated with polymerase and a sample was removed after 24% (d) and 70% (e) synthesis for electron microscopic studies (see Materials and Methods). The DNA samples are described in Fig. 2. In (e) the lengths of molecules with more than two ends were determined by summing the individual lengths of each branch. The ordinate represents the number of molecules of length μ_i × the length μ_i. The distribution therefore corresponds to length-average length distribution. See Table 1 for the number of molecules in each distribution.

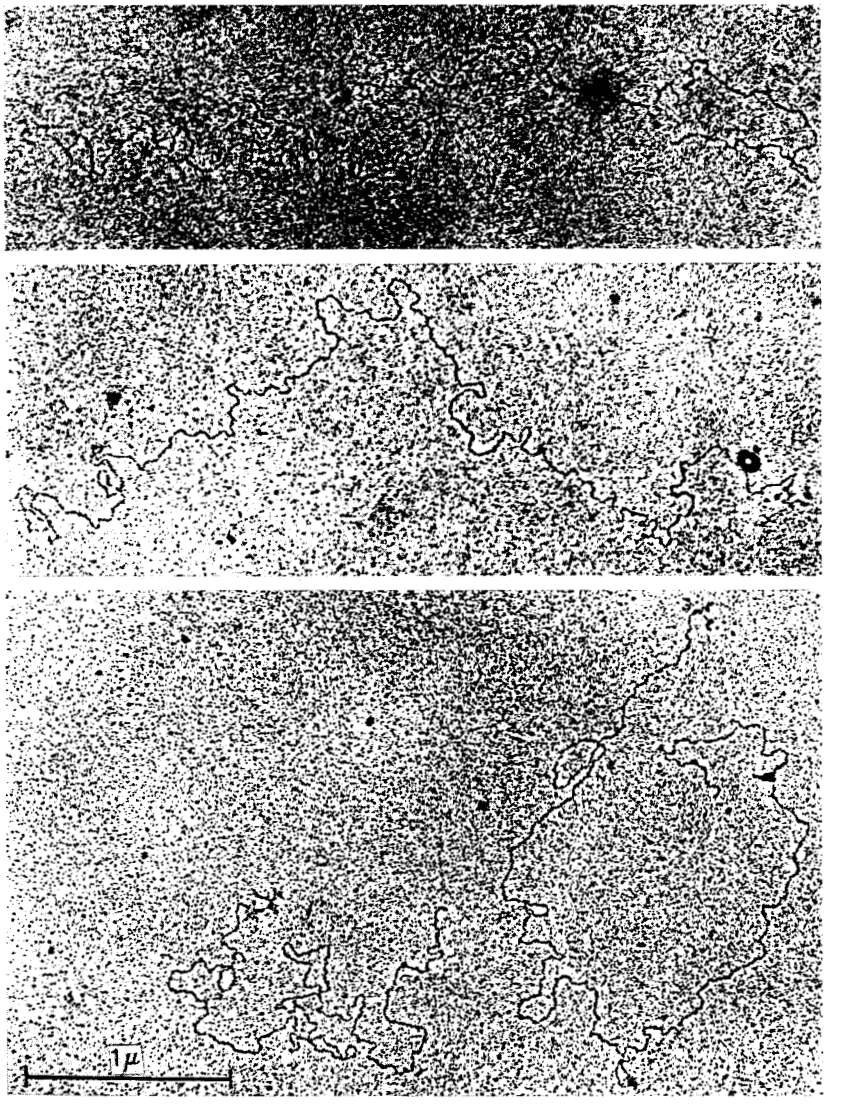

PLATE III. Electron micrographs of repaired DNA (24% synthesis of 29% degraded primer) showing: (a) increased helical length ($L_N = 7{\cdot}2\mu$); (b) a decrease in the number of puddles (see Table 1).

[*facing p. 54*

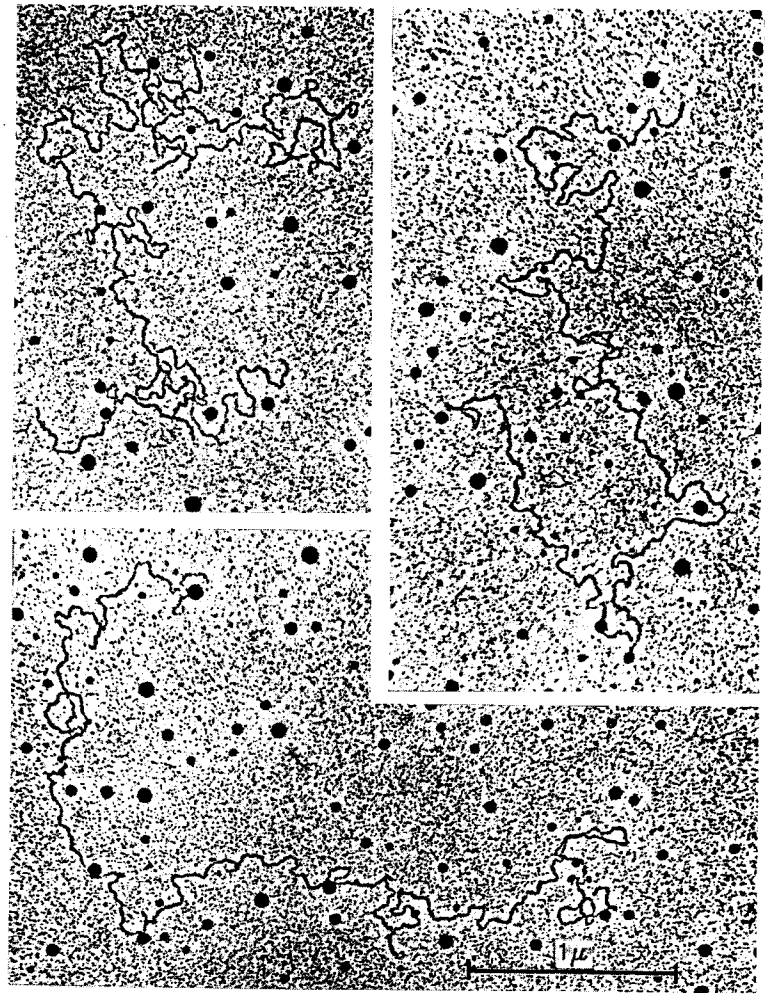

PLATE IV. Electron micrographs of product of extensive synthesis past the point of repair (70% synthesis of 29% degraded primer) showing: (a) increased helical length; (b) elimination of puddles; (c) the appearance of branching in many of the molecules (left, top micrograph).

The appearance of many black spherical objects in this specimen is not connected with DNA synthesis but rather arises from some unidentified impurity that appears in samples from time to time.

of the single-stranded regions of the primer should have been converted to double-stranded helical regions. According to the model (Fig. 1), the repaired molecules should have been 90% the length of the original undegraded T7 DNA (i.e. 8·2 μ). Plate III shows the appearance of repaired molecules, and Fig. 4(d) gives their length distribution. The distribution of lengths has increased as a result of repair (compare Fig. 4(c) and (d)); the number-average length has increased from 2·4 μ to 7·2 μ and the distribution has also become very broad. It should be noted that many molecules in this repaired sample correspond in length to that of the longer molecules in the native T7 DNA sample.

According to the model (Fig. 1), repair proceeds by conversion of the single strand into a double-stranded helix, and thus the length of the native section of the molecule should increase at the expense of material in a puddle. In agreement with this expectation, the puddles, which were numerous in the degraded primer, are decreased in the fully repaired sample (see Table 1).

Table 1 indicates that the repaired DNA almost always has only two ends per molecule, as opposed to the branched product observed when native DNA is used as primer (see below).

(iii) *Extensive synthesis with partially degraded T7 DNA as primer*

With 29% degraded DNA as primer, the polymerase reaction was carried to the extent of 70% synthesis. This is calculated to be a 41% increase in DNA beyond the point of complete repair of the partially single-stranded DNA. Plate IV shows micrographs of the DNA resulting from extensive synthesis, and Fig. 4(e) gives the distribution of lengths. There is a number of differences between this sample of DNA and that obtained when repair is the predominant component of the synthetic reaction. Table 1 indicates that the number of observable puddles has decreased; and further, that the number of molecules containing more than two ends, that is, with a branched structure, has increased to a degree typical of DNA synthesized from native primer (Richardson *et al.*, 1964*c*; Inman, Schildkraut & Kornberg, unpublished work). Although the number-average distribution of lengths has not increased greatly (Table 1), there has been an increase in the breadth of the length distribution to such a degree that now some of the molecules have a total length† that is longer than the original undegraded T7 DNA (compare Fig. 4(e) with (a) and (d)). If we ignore, for the moment, all molecules with more than two ends, we obtain the length distribution shown in Fig. 5(a); and it can be seen that there are no two-ended molecules with length greater than the original undegraded T7 DNA. In fact, the distribution is very similar to that given for T7 DNA (Fig. 4(a)). We conclude therefore that the super-length molecules indicated in Fig. 4(e) are longer because they have attached to them the DNA synthesized beyond the point of complete repair; it is also apparent that this attachment is in some way connected with the observed branching of these molecules. Figure 5(b) shows the distribution of the longest unbranched length of the branched molecules, and again we observe that the distribution does not extend to lengths greater than the original undegraded primer. Experiments to be described below indicate that the DNA synthesized after complete repair is not covalently linked to the primer; the linkage observed in the

† In the case of molecules with more than two ends, lengths were determined by adding together the individual lengths of each branch.

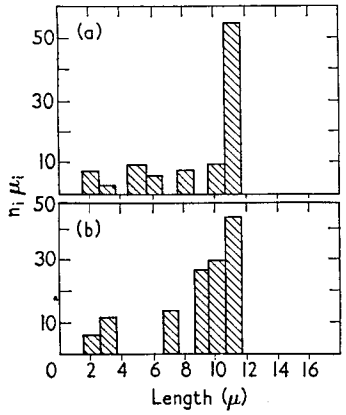

FIG. 5. *Distribution of lengths of branched and unbranched DNA resulting from* 70% *synthesis of* 29% *degraded DNA.* (See Fig. 4(e).)

(a) Lengths of the unbranched molecules; (b) longest continuous lengths of branched molecules. $n_i\mu_i$ is number of molecules of length $\mu_1 \times \mu_1$.

non-denatured material is, therefore, of some other kind, possibly by hydrogen bonds.

(c) *Density-gradient analysis*

(i) [$^{14}N^1H$]*T7 DNA as primer*

Native T7 DNA, 29% degraded by exonuclease III, was incubated with polymerase and samples were taken after 24, 40, 70 and 150% synthesis. These samples containing the original primer plus newly synthesized DNA were analysed for their buoyant density distribution in CsCl at neutral pH both before and after alkali-denaturation. The results are shown in Fig. 6.

(a) *Density gradient analysis before alkali-denaturation.* The native T7 DNA at the top of Fig. 6 (left column) formed a sharp band at a density of 1·710 g cm^{-3}. Upon degradation by exonuclease III (29%) there was a small but definite increase in density to 1·712 g cm^{-3}, a slight broadening of the band, and a skewing toward the heavier density. This conversion of the native DNA to that of a higher density by exonuclease action is consistent with the shift known to accompany denaturation of a native DNA; more extensive hydrolysis (more than 40%) has been shown to produce molecules banding at the density of completely single-stranded DNA (Richardson *et al.*, 1964*a*).

After 24% synthesis with polymerase, the repair process should have converted the single-stranded regions of the partially degraded molecules to double-stranded ones, according to the model proposed in Fig. 1. As seen in Fig. 6, the density of the peak returned to 1·710 g cm^{-3}, the density of double-stranded primer, and the peak became symmetrical about this density. Subsequent synthesis to 70 and 150% had no effect on either the density or the band profile. The latter amount of synthesis should not only have repaired the 29% degraded DNA but also resulted in a doubling of the DNA initially present.

(b) *Density-gradient analysis after alkali-denaturation.* Alkali-denaturation of the native DNA and the DNA treated with exonuclease III resulted in an increase of density to 1·724 g cm^{-3}, the value expected for the conversion of the helical molecule

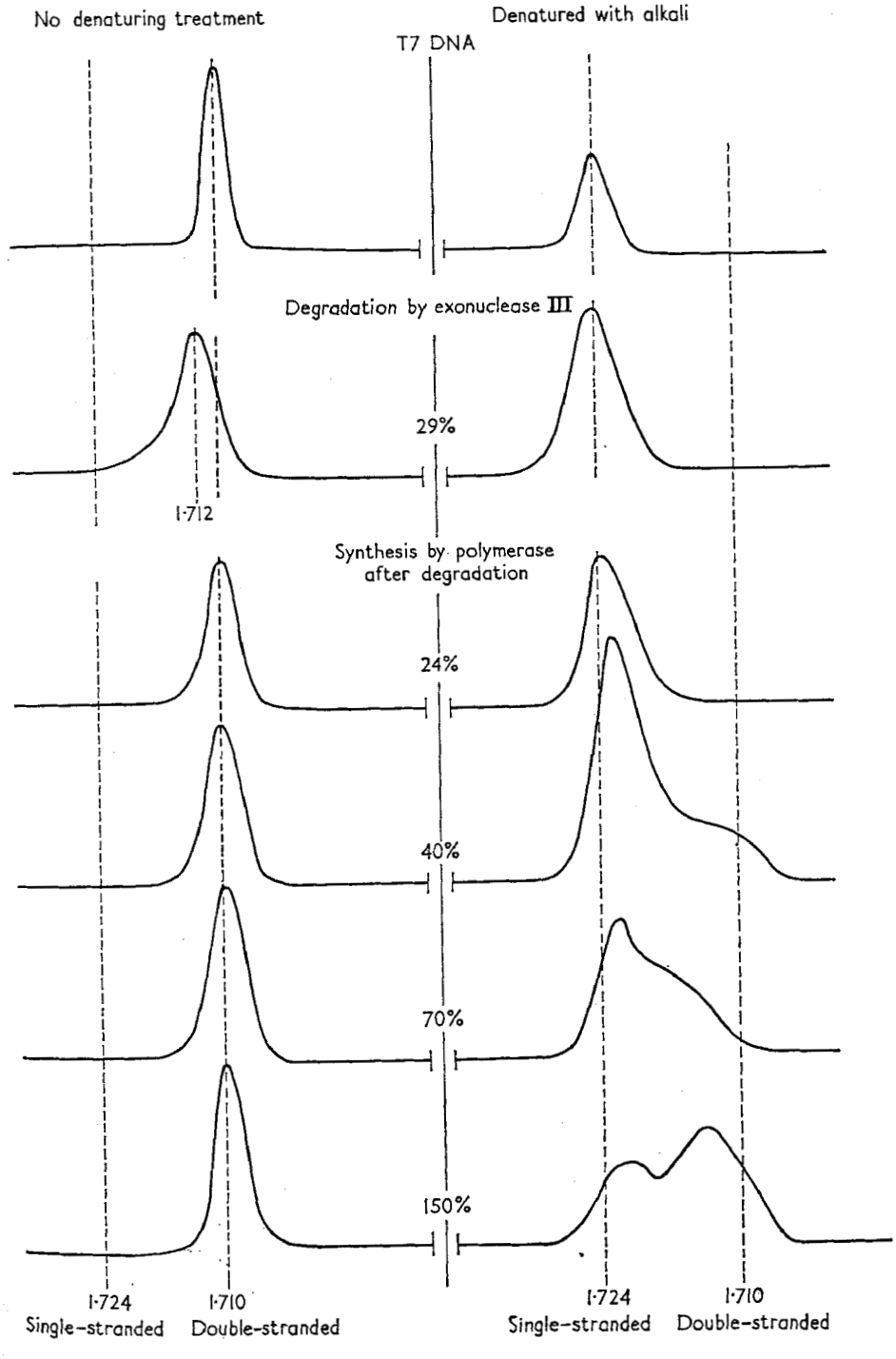

FIG. 6. *Density-gradient analysis of DNA synthesis primed by* [$^{14}N^{1}H$]*T7 DNA.* [$^{14}N^{1}H$]T7 DNA was degraded 29% by incubation with exonuclease III and the enzyme inactivated by heat as described in Materials and Methods. The partially degraded T7 DNA was incubated with polymerase (see Materials and Methods) and samples were removed after 24, 40, 70 and 150% synthesis. The reaction was stopped by the addition of 1/5 vol. of 10 × SSC, followed by dialysis

to a random coil (right column of Fig. 6). Denaturation of the 24% synthesis sample resulted in a single, unimodal peak at the expected density of single-stranded DNA (1·724 g cm^{-3}). However, at 40% synthesis, where synthesis has proceeded beyond repair, a peak appeared near the density of double-stranded DNA (1·710 g cm^{-3}). As synthesis progressed, this new peak increased in area, so that at 150% synthesis (121% beyond repair) there were two well-defined peaks, one near the density of single-stranded T7 DNA and one near the density of double-stranded T7 DNA.

These density-gradient results also support the model presented in Fig. 1. Treatment of the native T7 DNA with exonuclease, producing single-stranded regions in the DNA molecule, resulted in an increased density of the DNA in a CsCl density-gradient. Repair of these molecules restored the double-stranded structure and the density to that seen with native, double-stranded DNA. Furthermore, DNA synthesized during the repair process could be denatured and shown to band with single-stranded primer molecules. However, once repair of the degraded primer had been completed, DNA was synthesized which after alkali-treatment still banded in a CsCl density-gradient very near the density seen for the double-stranded DNA. This lack of denaturability of synthetic DNA made from a native primer by *E. coli* polymerase is described by Schildkraut *et al.* (1964), and will be discussed below. It is also significant that the DNA synthesized after repair formed bands distinct from the single-stranded primer after alkali-denaturation (as a result of its non-denaturability), indicating the absence of a covalent linkage between the product and primer (see also below).

(ii) *[$^{15}N^2H$]T7 DNA as primer*

The studies using [$^{14}N^1H$]T7 DNA as primer did not provide information as to the nature of the association between the primer and the product synthesized during repair, since the latter, when denatured by alkali-treatment, forms bands with the denatured primer whether covalently linked to it or not. To determine the linkage between primer and repaired product [$^{15}N^2H$]T7 DNA partially degraded to the extent of 25% by exonuclease III was prepared and used as primer. The partially single-stranded DNA was incubated with polymerase and [$^{14}N^1H$]deoxyribonucleoside triphosphates and samples were taken at 12, 19, 35 and 62% synthesis. These samples, containing the original [$^{15}N^2H$]primer plus the newly synthesized [$^{14}N^1H$] product, were analysed for their buoyant density distribution in CsCl at neutral pH both before and after alkali-denaturation. The results are shown in Fig. 7.

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against 0·20 M-NaCl containing 0·002 M-tris–HCl buffer, pH 7·8, and finally against 0·02 M-NaCl containing 0·002 M-tris–HCl, pH 7·8. After dialysis, samples were centrifuged in CsCl as described in Materials and Methods. The microdensitometer tracings are shown in the left column for the samples with no denaturing treatment, and in the right column for the alkali-denatured samples. The latter were prepared by the addition of 10 μmoles of NaOH to a solution containing 5 to 10 mμmoles of DNA and 100 μmoles of NaCl in a volume of 0·50 ml. After 30 min at room temperature, 300 μmoles of tris–HCl buffer, pH 7·2, were added, followed by 10 μmoles of HCl. CsCl was added to the desired concentration and the solution centrifuged as described in Materials and Methods.

Each cell contained from 5 to 10 mμmoles of DNA (primer plus product). The DNA degraded by exonuclease III was dialysed prior to centrifugation, first against 0·20 M-NaCl containing 0·002 M-tris, pH 7·8, and then against 0·02 M-NaCl containing 0·002 M-tris, pH 7·8. The density of alkali-denatured T7 DNA is indicated in the left column for reference, as is also the density of native T7 DNA in the right column. [$^{15}N^2H$]*E. coli* DNA (ρ = 1·764 g cm^{-3}) was used as a standard for density determinations.

(a) *Density-gradient analysis before alkali-denaturation.* The native [$^{15}N^2H$]T7 DNA at the top of Fig. 7 (left column) formed a sharp band at a density of 1·758 g cm^{-3}. Upon degradation by exonuclease III (25%), there was an increase in density of 0·004 g cm^{-3}, presumably due to the formation of single-stranded regions as observed after similar treatment of the [$^{14}N^1H$]T7 DNA (Fig. 6).

After 12% synthesis with polymerase and [$^{14}N^1H$]deoxynucleoside triphosphates, the [$^{15}N^2H$]DNA band disappeared and a somewhat broader band of lower density appeared. As synthesis progressed, the density of this band became progressively less and broader, resulting in a mean density of 1·733 g cm^{-3} after 62% synthesis. The mean densities of the bands observed during synthesis (Fig. 7) are at positions expected from the extent of replication, assuming that the primer and product in the complex contribute independently to the density. An association of primer with product even after synthesis has proceeded for several replications of DNA has been previously described (Richardson *et al.*, 1963; Richardson *et al.*, 1964*c*; Schildkraut *et al.*, 1964).

(b) *Density-gradient analysis after alkali-denaturation.* Each of the DNA samples shown in the left column of Fig. 7 was denatured with alkali and then examined in a CsCl density-gradient at neutral pH (right column of Fig. 7). Alkali-denaturation of the native DNA and the DNA treated with exonuclease III resulted in an increase of density to 1·773 g cm^{-3}, as expected for the conversion of a helical molecule to a random coil. Upon denaturation of the 12% synthesis product, a broader band appeared which was skewed toward the lower density. At 19 and 35% synthesis, a range of synthesis (repair) which should encompass the extent of degradation, there was an increase in the asymmetry of the band, with an increase in the DNA at a hybrid density; the peak density was shifted to 1·770 g cm^{-3}. There was no detectable DNA appearing at the density of either single-stranded or double-stranded [$^{14}N^1H$]T7 DNA. However, after 62% synthesis, an amount which is in considerable excess for the repair phase of synthesis, denaturation gave rise to two distinct peaks. The heavier material, at a density of 1·769 g cm^{-3}, gave an asymmetrical peak and contained DNA at hybrid density; whereas the lighter material had a density of 1·712 g cm^{-3}. The latter density is similar to that seen with double-stranded [$^{14}N^1H$]T7 DNA (1·710 g cm^{-3}).

When native [$^{15}N^2H$]T7 DNA was used to prime a 20% synthesis, an amount similar to that necessary to repair the degraded [$^{15}N^2H$]T7 DNA primer, alkali-denaturation of the product gave rise to two bands in a CsCl density-gradient. One band, at a density of 1·773 g cm^{-3}, corresponded to single-stranded [$^{15}N^2H$]T7 DNA and did not demonstrate molecules at hybrid density as seen in Fig. 7 with the partially degraded DNA as primer. The other band, at a density of 1·713 g cm^{-3}, demonstrated that the [$^{14}N^1H$]product was non-denaturable and not covalently attached to the primer. The formation by the non-denaturable [$^{14}N^1H$]product of a band separate from the [$^{15}N^2H$]primer after 20% synthesis with a native DNA primer is to be contrasted with the formation of hybrid molecules after a similar amount of synthesis with a partially degraded DNA primer (Fig. 7).

The use of degraded [$^{15}N^2H$]T7 DNA as primer supports the previous studies (Richardson *et al.*, 1963; Richardson *et al.*, 1964*c*; Schildkraut *et al.*, 1964) with a native [$^{15}N^2H$]*B. subtilis* DNA primer; that is, the bands show a progressive conversion of the primer to a hybrid of intermediate density. After 62% synthesis, all of the primer was incorporated into a major peak with a buoyant density anticipated from

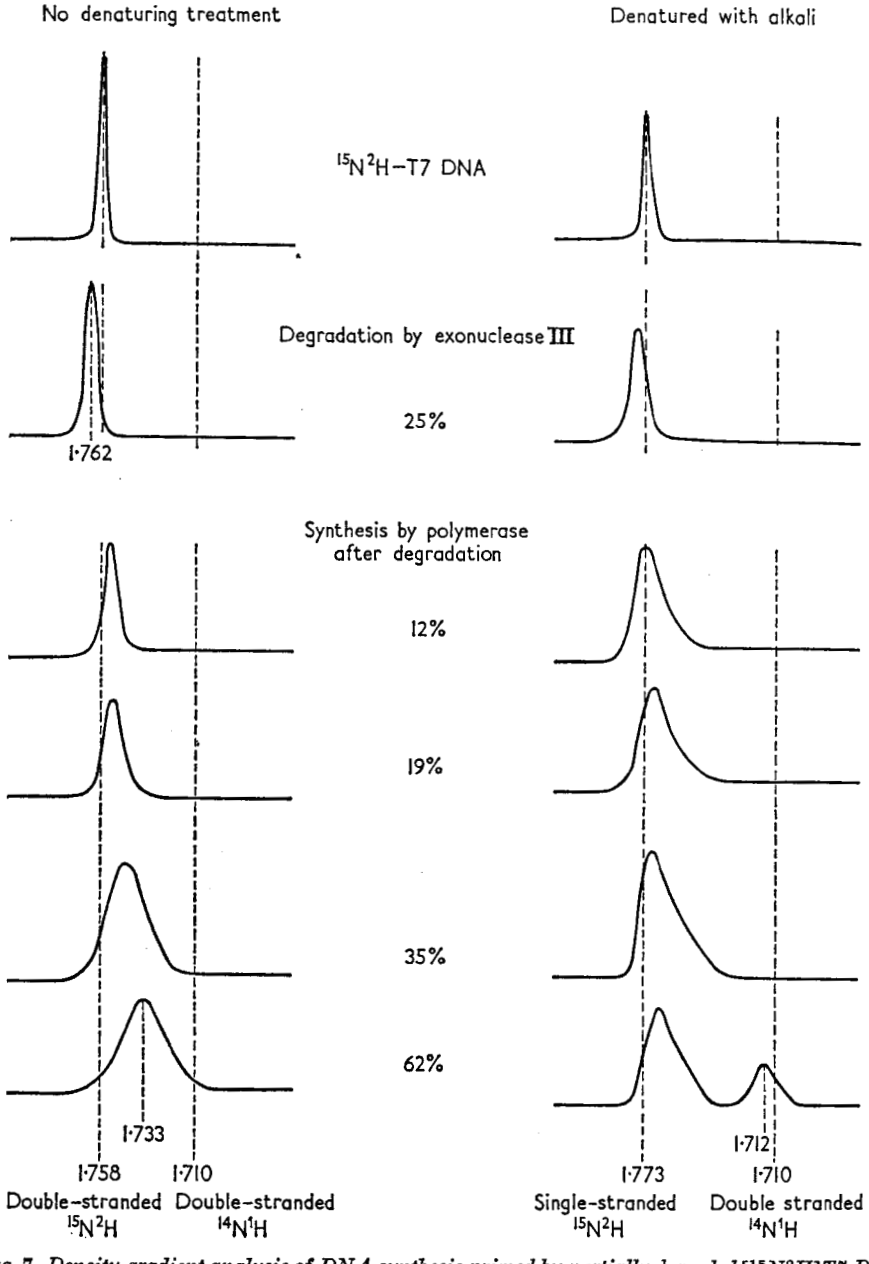

FIG. 7. *Density-gradient analysis of DNA synthesis primed by partially degraded* [$^{15}N^{2}H$]*T7 DNA.* [$^{15}N^{2}H$]T7 DNA was degraded 25% by exonuclease III, incubated with polymerase and analysed essentially as described for the [$^{14}N^{1}H$]T7 DNA in Fig. 6. The density of native [$^{14}N^{1}H$]T7 DNA is indicated for reference. *Tetrahymena pyriformis* DNA ($\rho = 1{\cdot}684$ g cm^{-3}) was used as a standard for density determinations.

the composition of the light and heavy atoms. Furthermore, denaturation of the 62% synthesis product produced two peaks, one of which resembled that due to the [$^{14}N^{1}H$]double-stranded product. The appearance of non-denaturable product as a result of extensive synthesis with native DNA is described in detail by Schildkraut *et al.* (1964). However, what had not been previously observed with a native DNA primer was the presence of DNA of hybrid density following denaturation of the product. While a discrete hybrid band was not obtained, nevertheless the appearance of DNA at a hybrid density following denaturation strongly supports the argument that DNA synthesized during the initial rapid rate of synthesis (Fig. 2) is covalently linked to the primer molecule. Subsequent to this repair process, DNA synthesis appears to be similar to that seen with the native DNA as primer, producing a non-denaturable [$^{14}N^{1}H$] product (see below).

(iii) *Linkage of product to primer by a mixed ribo-, deoxyribonucleotide polymer*

The ability of *E. coli* DNA polymerase to synthesize a mixed polymer of ribo- and deoxyribonucleotides (Berg *et al.*, 1963) provided an additional means of studying the association of degraded primer DNA with the DNA synthesized during repair.

$^{15}N^{2}H$-labeled *B. subtilis* DNA was degraded 30% by exonuclease III. This partially degraded DNA was then used as a primer in the DNA polymerase system, with rCTP replacing dCTP among the four deoxynucleoside triphosphates and Mn^{2+} replacing Mg^{2+}. The reaction was allowed to proceed only to the extent of 2% synthesis, assuming that synthesis of the mixed polymer would be covalently attached to the 3′-hydroxyl terminus of the primer, as shown in Fig. 8. After removal of the rCTP and Mn^{2+} by dialysis, 23% additional synthesis was obtained in the standard polymerase reaction. This should result in [$^{14}N^{1}H$]product covalently linked to the [$^{15}N^{2}H$]primer through a short sequence of ribo- and deoxyribonucleotides as postulated in Fig. 8.

Density-gradient analysis of these products, along with native [$^{14}N^{1}H$] and [$^{15}N^{2}H$]*B. subtilis* DNA included for reference, are shown in Fig. 9. As in the case of the T7 DNA, there was an increase in density of the [$^{15}N^{2}H$]DNA after exonuclease treatment. Synthesis of the mixed ribo- and deoxyribonucleotide polymer to the extent of 2% resulted in no appreciable change in the band profile of the degraded DNA. Further synthesis with [$^{14}N^{1}H$]deoxyribonucleotides to 23% led to the expected decrease in density of the hybrid product from 1·755 g cm^{-3} to 1·736 g cm^{-3}.

When the 23% synthesis-product was denatured by mild alkaline treatment (0·02 M-NaOH at 25°C for 15 minutes) a marked broadening of the peak occurred (Fig. 8). Presumably this denatured DNA includes single-stranded [$^{15}N^{2}H$]molecules and single-stranded [$^{14}N^{1}H$]molecules and, more significantly, a large number of molecules of hybrid density. Identical band profiles were observed after denaturation by heating (at pH 7·4, five minutes at 100°C and quickly cooling) or formamide treatment (dialysis against 97% formamide at room temperature).

When the same product was subjected to strong alkaline treatment (0·05 M-NaOH for 90 minutes at 37°C) sufficient to hydrolyse ribose phosphodiester bonds, it was resolved into products giving distinct peaks (Fig. 9). The peak at higher density was at the density (1·766 g cm^{-3}) of single-stranded [$^{15}N^{2}H$]*B. subtilis* DNA, and the peak at lower density (1·720 g cm^{-3}) at that of single-stranded [$^{14}N^{1}H$]*B. subtilis* DNA. The same results were obtained by heating the product at 100°C in 0·05 M-NaOH and then quickly cooling and neutralizing. Alkaline hydrolysis strong enough

to break all ribonucleotide linkages (0·20 M-NaOH at 37°C for 15 hours) produced the same separation of product and primer. This latter treatment of a 19% synthesis product produced in a standard polymerase reaction primed by partially degraded [$^{15}N^2H$]DNA but without rCTP did not lead to a separation of the primer–product complex in the hybrid region (Fig. 7).

These results indicate that the [$^{14}N^1H$]product was covalently linked, through a mixed ribo- and deoxyribonucleotide polymer, to the [$^{15}N^2H$]primer as postulated in Fig. 8. Denaturation by heat, formamide or mild alkaline treatment resulted in

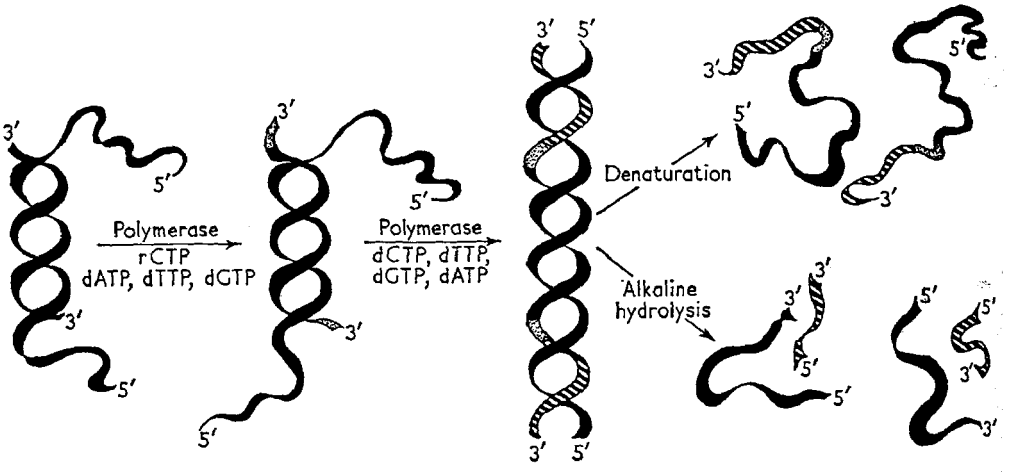

FIG. 8. *Postulated scheme for covalent linkage of synthesized DNA to partially degraded DNA through a sequence containing ribo- and deoxyribonucleotides.* DNA polymerase, when incubated with a partially degraded [$^{15}N^2H$]DNA in the presence of rCTP, dATP, dTTP, dGTP and Mn^{2+}, covalently adds a short nucleotide sequence, containing interspersed cytosine ribonucleotides (stippled segment). Completion of repair by polymerase with the 4 deoxyribonucleotides results in the covalent linkage of this product (striped segment) to primer through the stippled segment. Denaturation of the repaired DNA by heat or mild alkaline treatment separates the two strands but preserves the linkage of product to primer. Hydrolysis of the ribonucleotide linkages by alkaline treatment results in the release of the [$^{14}N^1H$]product from the [$^{15}N^2H$]primer and permits their separation in a CsCl density-gradient.

separation of the strands of the repaired molecules without disrupting this linkage in at least some of the molecules. However, more vigorous alkaline treatment, sufficient to hydrolyse one or more ribose phosphodiester bonds per strand, resulted in the complete separation of the primer from the product. As further support for this conclusion, a preparative CsCl density-gradient of such an alkaline digest demonstrated that the material giving ^3H (more than 90%) counts in the primer formed a band at the density of single-stranded [$^{15}N^2H$]*B. subtilis* DNA and that the material giving ^{14}C counts (more than 90%) in the product formed a band at a density of single-stranded [$^{14}N^1H$]*B. subtilis* DNA. There was no evidence of a hybrid peak. The presence of DNA at the densities of both [$^{14}N^1H$]product and [$^{15}N^2H$]primer after denaturation by heat, formamide or mild alkaline treatment could arise either from phosphodiester bond cleavage during the course of the two synthetic reactions or during the denaturation. Whether the presence of these species is a result of synthesis not initiated by a covalent attachment to primer requires additional study under a variety of conditions.

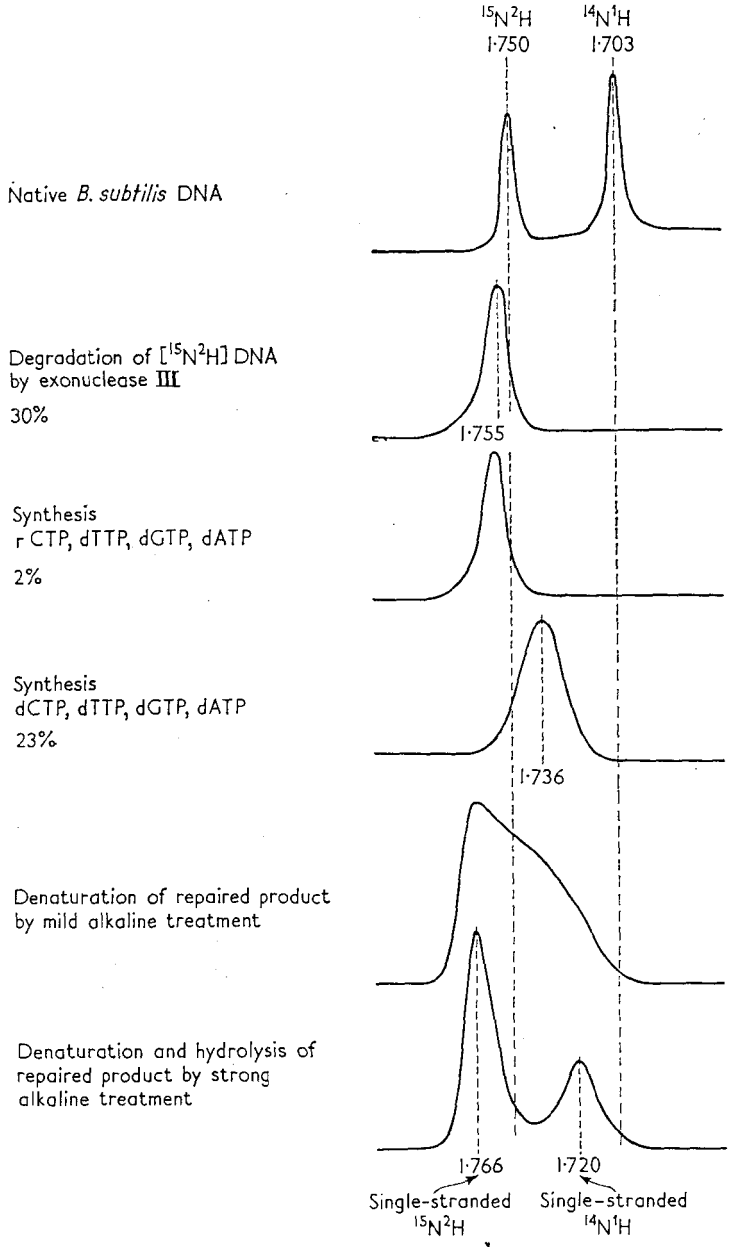

FIG. 9. *Density-gradient analysis of the* [$^{14}N^{1}H$]*product attached to a partially degraded* [$^{15}N^{2}H$]B. subtilis *DNA primer through a ribo-, deoxyribonucleotide mixed polymer.* [$^{15}N^{2}H$]*B. subtilis* DNA was degraded 29% by incubation with exonuclease III. The reaction mixture (4·0 ml.) contained 2 μmoles of DNA, 280 μmoles of tris–HCl buffer, pH 8·0, 4 μmoles of $MgCl_2$, 40 μmoles of 2-mercaptoethanol, and 450 units of exonuclease III. Incubation was at 37°C. The reaction was stopped and the extent of degradation was determined as described in Materials and Methods.

The partially degraded DNA was incubated with polymerase under the conditions described in Materials and Methods for the synthesis of a mixed ribo-, deoxyribonucleotide polymer containing rCTP. The reaction was stopped after 2% synthesis by the addition of 1/5 vol. of 10 × SSC and polymerase was inactivated by heating at 65°C for 10 min. The reaction mixture was dialysed for 36 hr against three changes of buffer (0·20 M-NaCl containing 0·002 M-tris–HCl buffer, pH 7·4) and then against 0·02 M-NaCl containing 0·002 M-tris–HCl, pH 7·4, at 4°C.

(d) *Denaturability of product*

The facility with which DNA synthesized by polymerase with a native primer renatures following denaturation has been described elsewhere (Schildkraut *et al.*, 1964). The following results indicate that, with a partially single-stranded primer, the product synthesized during repair is denaturable, but the DNA synthesized subsequent to repair is non-denaturable.

(i) *Density-gradient analysis*

When a 29% degraded T7 DNA served as primer for 24% synthesis, both the primer and product, after alkali-denaturation, increased in density to that expected for denatured T7 DNA (Fig. 6). The products of more extensive synthesis to levels of 40, 70 and 150% gave rise to DNA which remained at the density of double-stranded T7 DNA after alkali-denaturation (Fig. 6). The formation of this non-denaturable DNA, not covalently linked to the primer, is more clearly seen from Fig. 7, where partially degraded [$^{15}N^{2}H$]T7 DNA served as primer. That the product of polymerase synthesis does not have to be attached to the primer to remain single-stranded is demonstrated by the experiment described in Fig. 9. The product covalently attached to the primer through a mixed polymer of ribo- and deoxyribonucleotides during repair was dissociated from the primer and formed a band in a CsCl density-gradient at the density of denatured DNA.

(ii) *Susceptibility to exonuclease I*

Additional evidence for the denaturability of the DNA synthesized during repair and the non-denaturability of the DNA synthesized subsequent to repair is provided by the susceptibility of the product to exonuclease I. When native T7 DNA was used to prime a 30% synthesis, only 5% of the product was found to be denaturable, as determined by its susceptibility to exonuclease I after alkali-treatment. However, if a 29% degraded T7 DNA was used to prime a 30% synthesis, approximately 80% of the product was denatured by alkali-treatment. If synthesis in the latter case continued to 90%, then only 33% of the product was denaturable. These results are interpreted to mean that the DNA synthesized early under the direction of a single-stranded template and covalently attached to it is denaturable; but that as synthesis continues, a large proportion of the product is contained in strands of the type primed by native DNA templates (Schildkraut *et al.*, 1964) and is non-denaturable.

The dialysed DNA solution was incubated with polymerase and the extent of synthesis was assayed as described in Materials and Methods. The DNA concentration in the reaction mixture was increased to 69 mμmoles/ml. and the deoxynucleoside triphosphates and polymerase were increased proportionately. At 23% synthesis the reaction was stopped by the addition of 10 × SSC and dialysed as described above.

The 23% synthesis product was denatured by incubating 30 mμmoles of DNA (primer plus product) with 0·02 M-NaOH, 0·10 M-NaCl in a volume of 0·50 ml. at 25°C for 15 min. The solution was neutralized by the addition of 30 μmoles of tris–HCl buffer, pH 7·2, and 10 μmoles of HCl. Denaturation and hydrolysis of the ribonucleotide linkage was carried out by incubating 30 mμmoles of DNA (primer plus product) with 0·05 M-NaOH, 0·10 M-NaCl in a volume of 0·50 ml. at 37°C for 90 min. The solution was neutralized by the addition of 30 μmoles of tris–HCl buffer, pH 7·2, and 25 μmoles of HCl.

Each cell contained from 5 to 15 mμmoles of DNA (primer plus product), except for the lower two tracings which contained 30 mμmoles of DNA. The DNA degraded by exonuclease III was dialysed first against 0·20 M-NaCl containing 0·002 M-tris–HCl buffer, pH 7·8, and then against 0·02 M-NaCl containing 0·002 M-tris–HCl buffer, pH 7·8. *Tetrahymena pyriformis* DNA ($\rho = 1{\cdot}684$ g cm^{-3}) was used as a standard for density determinations.

If this interpretation be correct, then exonuclease I should provide a ready quantitative assay for the amount of repair which has occurred at any time during synthesis. This is shown to be the case in Fig. 10. If native T7 DNA was used to prime a polymerase reaction, the product obtained was only 3% repaired, as determined by its susceptibility to exonuclease I. With a 10% degraded T7 DNA as primer, the repair reached a limit of 10%; and with a 29% degraded DNA the limit for repair was 30%. This assay provides additional information concerning the kinetics

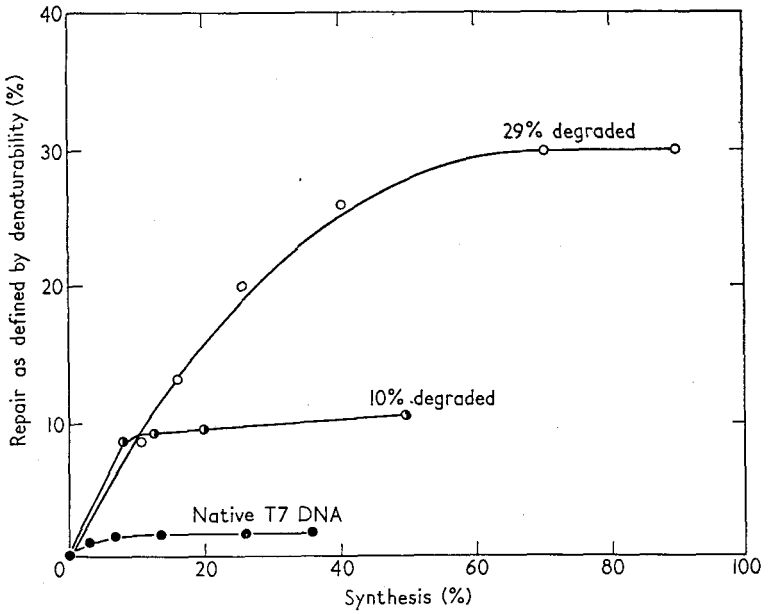

FIG. 10. *Repair as measured by denaturability of product.* T7 DNA, untreated, 10% and 29% degraded (see legend to Fig. 2) was incubated with DNA polymerase (see Materials and Methods) and samples were removed after varying extents of synthesis as indicated. Polymerase was inactivated by the addition of 1/5 vol. of 10 × SSC and heating at 65°C for 10 min. After dialysis against 0·02 M-NaCl containing 0·002 M-tris–HCl, pH 7·8, 0·8 mμmole of the DNA (primer plus product) was denatured with alkali by making the solution 0·02 N with NaOH for 30 min at room temperature and then neutralizing with HCl. The alkali-denatured DNA was incubated with exonuclease I and the susceptibility of the ^{14}C radioactivity in the product to exonuclease I was determined as described in Materials and Methods. The mμmoles of ^{14}C-product susceptible to exonuclease I divided by the mμmoles of DNA originally present before degradation by exonuclease III yields the "percentage repair as defined by denaturability". Control incubation of DNA samples not treated with alkali showed that less than 10% of the product was susceptible to exonuclease I.

of repair. It is apparent from Fig. 10 that the 29% degraded DNA required approximately 50% synthesis before the repair was complete. Thus, the repair process leading to denaturable DNA and the synthesis of the new, non-denaturable chains appear to overlap in time; the non-linear portion of the biphasic kinetic curves shown in Fig. 2 is further evidence for this. The small amount of denaturable DNA product produced by a native DNA-primed reaction suggests that 3% of this DNA preparation was single-stranded. It is of interest, therefore, that with this same sample of native T7 DNA as primer in a polymerase reaction incubated at 20°C (Fig. 3), a temperature at which presumably only the copying of single-stranded regions in the primer occurs, synthesis also reached a limit of approximately 3%.

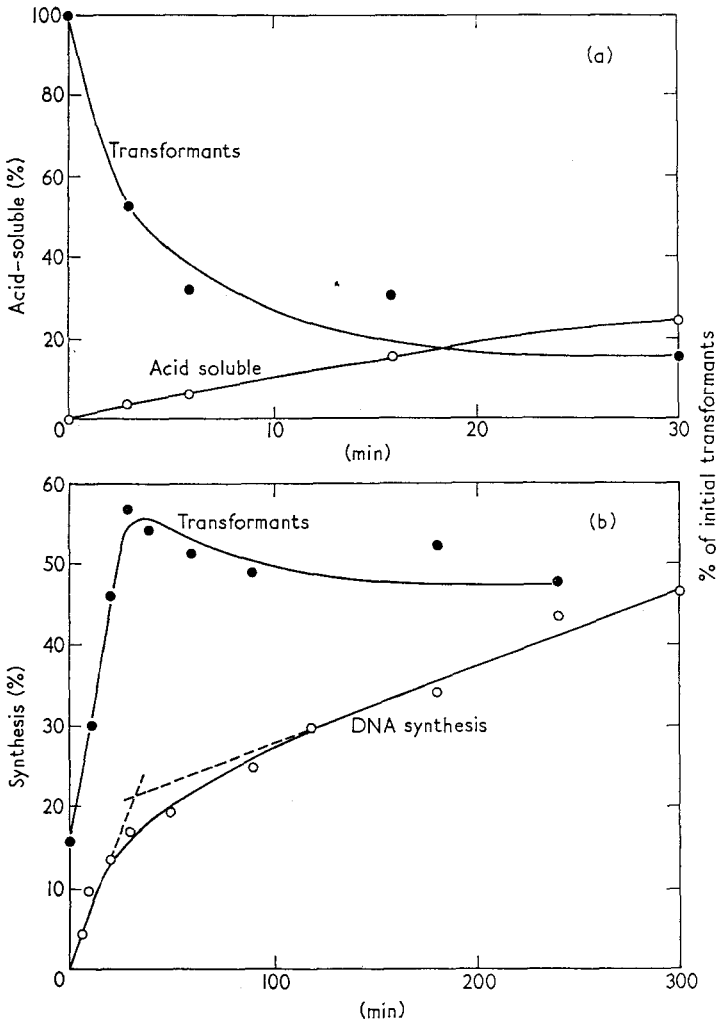

FIG. 11. *Effect of degradation and repair on the genetic activity of* B. subtilis *DNA. B. subtilis* (SB-19) DNA was incubated with exonuclease III and the acid-soluble radioactive material was measured as described in Materials and Methods. The reaction was stopped after 24% synthesis by heating (see Materials and Methods) and the degraded DNA was immediately used to prime a polymerase reaction. The polymerase reaction and the determination of the extent of synthesis were as described in Materials and Methods.

At the times indicated, 0·005-ml. samples of the exonuclease-treated reaction mixture containing 1·4 mμmoles of DNA were removed and added to culture tubes containing 0·10 ml. of 0·10 M-sodium citrate. Zero time was before the addition of enzyme. During the polymerase reaction, 0·02 ml. of the reaction mixture containing 0·6 mμmole of DNA primer (based on the DNA before degradation) was removed and added to a culture tube containing 0·10 ml. of 0·10 M-sodium citrate. Zero time was before the addition of enzyme. Transformation assays for the try_2^+ (indole) marker were as described in Materials and Methods. Samples taken before and after heat inactivation of the exonuclease gave essentially the same number of transformants. The number of transformants per mμmole of DNA at zero time in the polymerase reaction mixture was the same as after 24% degradation in the exonuclease reaction mixture. A control tube of 24% degraded DNA lacking polymerase showed no change in the number of transformants during the same period of incubation.

(e) *Effect of degradation and repair on genetic activity of* B. subtilis *DNA*

When [^{3}H]*B. subtilis* DNA was incubated with exonuclease III, there was a decrease in the number of transformants as measured by transformation of the try_2^+ (indole) marker (Fig. 11(a)); at 24% degradation, the number of transformants was only 16% of those initially present. This decrease in the genetic activity might be the result of removal of the try_2^+ region of the DNA molecule, the decreased incorporation of the partially single-stranded DNA into the bacterial cells, or both. In any event, the use of this degraded DNA in the polymerase system provided an additional means of comparing the properties of the DNA synthesized during the repair phase with those of DNA produced during extensive synthesis of new strands.

Incubation of the degraded DNA with polymerase resulted in synthesis, with a rapid phase corresponding roughly to the extent of degradation (Fig. 11(b)). As in the case of the degraded T7 DNA, this rapid rate of synthesis was followed by a slower rate which was then permitted to continue to the extent of 46% synthesis. As shown in Fig. 11(b), there was an increase in the number of transformants to a level of 55% of that measured with the untreated, native DNA. A control incubation of degraded DNA without polymerase showed no change in the number of transformants during the same interval. Of particular interest was the correspondence of the restoration of genetic activity to the rapid phase of synthesis; subsequent to this phase, there was no further increase in transformants. Preliminary studies (Josse, Kornberg, Marmur, Schildkraut & Doty, 1962) with heat-denatured DNA as a primer showed large increases in genetic activity concomitant with enzyme synthesis. However, as described previously (Richardson *et al.*, 1963), extensive synthesis (100 to 500%) primed by native *B. subtilis* DNA leads to no increase in genetic activity. These results suggest that restoration of the helical structure accounts for the recovery of genetic activity in the degraded DNA, but do not provide evidence that the newly synthesized DNA itself contains genetic activity.

4. Discussion

Our views on the action of nuclease and polymerase depend in large measure on our knowledge of DNA structure. These enzymes, when better understood, serve in turn as reagents for further analysis and manipulation of DNA structure. The results of this investigation illustrate these points. Native DNA degraded by exonuclease III to a rather uniform population of partially single-stranded molecules is then restored to its original size and state through stepwise replication by polymerase (Fig. 1).

Earlier deductions about the nature and specificity of exonuclease III action (Richardson *et al.*, 1964*a*) have been confirmed. Starting from each 3′-hydroxyl end of the bihelical molecule, there is a progressive denudation, leaving unmatched single strands and a shortened helix. Electron micrographs display these residual single strands in what appear as puddle-like regions; measurements of helical length show a reduction corresponding roughly to the extent of degradation deduced by chemical analysis. An increased buoyant density and a loss of genetic activity support this picture of the exonuclease-degradation product. Provided with this partially single-stranded molecule as primer, polymerase action has been revealed in a relatively uncomplicated fashion. Replication starts by covalent linkage to each 3′-hydroxyl terminus, with the single strands serving as templates to dictate the

sequential assembly of nucleotides. The rate of this repair process is at least five times as great as that seen with native DNA as primer and proceeds readily at 20°C, a temperature at which the native DNA fails to promote replication. With the replacement of nucleotides removed in the first instance by exonuclease, there is a partial recovery of genetic activity, a decrease in buoyant density and, as judged by electron microscopy, the original helical lengths are restored and the puddle-like regions erased.

The phase of synthesis that succeeds the stage of repair is also of considerable interest, since it can be so readily identified with characteristics of replication primed by native DNA templates (Schildkraut *et al.*, 1964). Upon reaching the point at which repair of the exonuclease degradation has been completed, the rate of synthesis drops sharply to that observed with native DNA. Subsequent synthesis is incorporated into a product which, like that primed by native DNA, is not covalently linked to the primer and displays two unusual properties: a capacity to recover a helical structure after denaturing treatments, and a branched appearance in the electron microscope. These results, with others described in the preceding paper (Schildkraut *et al.*, 1964), suggest that abnormalities introduced into enzymically synthesized DNA are not primarily an aberration of polymerase action but rather a consequence of complications in the structure and orientation of the templates.

This work has benefited greatly from electron microscope measurements and has in turn demonstrated how useful this technique can be in determining the length of DNA molecules and distinguishing between helical and coiled regions of a molecule. These analyses, together with kinetic measurements of synthesis at different temperatures and determinations of denaturability of the synthetic product, reveal the heterogeneity and complexities which are encountered when even so small and simple a primer as T7 DNA is used.

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